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Circulating endothelial cells in Kawasaki disease

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SUMMARY

Recent reports have demonstrated that circulating endothelial cells (CECs) are observed in several diseases with vascular injury. Because Kawasaki disease (KD) is one type of systemic vasculitis, we hypothesized that an increased number of CECs may be associated with the appearance of complicated coronary artery lesions (CAL). In the present study we investigated the enumeration and origin of CECs in 20 patients with KD, using an immunohistochemical method with monoclonal antibodies: clone P1H12 against ECs and clone AC133 against endothelial progenitor cells (EPCs), which were derived from the bone marrow. The mean number of CECs increased significantly ($P < 0{\cdot}05$) from the acute through the subacute phases of KD compared with both the convalescent phase of KD and healthy children. The mean number of CECs was significantly ($P < 0{\cdot}05$) higher in six KD patients with CAL than in 14 KD patients without CAL. The population of EPCs in the total CECs in KD was $4{\cdot}4 \pm 1{\cdot}2\%$ (range 0–18%). The number of EPCs during the subacute phase was also significantly higher ($P < 0{\cdot}05$) in KD patients with CAL than in those without CAL. Our findings indicate that the number of CECs increase in KD vasculitis and suggest that the increased numbers of CECs and EPCs may reflect the EC damage of this disease.

Keywords circulating endothelial cells endothelial progenitor cell Kawasaki disease

INTRODUCTION

Endothelial cells (ECs) play a crucial role in the physiological and pathological processes of haemostasis, inflammation and angiogenesis [1]. Maintaining EC integrity plays an important role in preserving vascular homeostasis, especially regarding appropriate thromboresistance and barrier function. ECs consist of a huge population of cells and respond to several stimulators including infectious agents, proinflammatory cytokines, growth factors and oxidative stress [1]. Certain pathological situations are considered to induce EC detachment from the vascular walls into the circulation [2]. Several investigators have reported that circulating ECs (CECs) were found in various conditions with vascular injury such as thrombotic thrombocytopenic purpura [3], Mediterranean spotted fever [4], cytomegalovirus infection [5], sickle cell anaemia [6], acute myocardial infarction [7] and systemic lupus erythematosus [8]. These studies also revealed that an increased number of CECs is a useful marker of vascular injury.

Recently, Asahara *et al*. [9] discovered the existence of circulating endothelial progenitor cells (EPCs) in human peripheral blood, and the cells are believed to originate from the bone marrow. Reportedly, EPCs are abundant in umbilical cord blood [10] and are mobilized into the circulation in patients with acute myocardial infarction [11]. The recruitment of EPCs from the circulation is thought to contribute to the postnatal physiological and pathological neovascularization *in vivo* [12,13]. Recently, bone marrow-derived immature EPCs have been reported to differ from vessel-derived mature ECs, using a monoclonal antibody (MoAb, clone AC133) against the specific surface marker for EPCs [14,15].

Kawasaki disease (KD) is an acute febrile illness that affects predominantly infants and children [16]. This disease is characterized as one type of systemic vasculitis which may have coronary artery involvement [17]. The immune system shows marked activation during the acute phase of KD, thus suggesting that activated effector cells induce an increased production of cytokine, including IFN-γ, IL-1, IL-6 and TNF-α, which can cause endothelial cell activation and damage [18]. In the present study, we investigated the enumeration and origin of CECs in patients with KD using an immunohistochemical method with MoAbs.

METHODS

Reagents

The following reagents were prepared: murine MoAb against EC (clone P1H12) from Chemicon International Inc., Temecula, CA,

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USA; murine MoAb against VE-cadherin (clone TEA1/31), MoAb against E-selectin (clone 1·2B6) and fluorescein isothiocyanate (FITC)-labelled goat antimouse immunoglobulin (Ig) from Dako, Glostrup, Denmark; phycoerythrin (PE)-labelled murine MoAb against AC133 (clone AC133/1) from Militenyi Biotech, Bergisch Gladbach, Germany; sheep antimouse Ig-carried magnetic beads from Dynal, Oslo, Norway; and 4′-6-diamidino-2-phenylindole (DAPI) from Molecular Probes Inc., Eugene, OR, USA. P1H12 responds to CD146 antigen, which is expressed on the surface of ECs [19]. AC133 responds to a surface antigen on EPCs, but this MoAb does not respond to mature ECs [14,15]. As negative and positive controls for P1H12, we used peripheral lymphocytes and human umbilical vein ECs (HUVECs), respectively. As negative and positive controls for AC133, we used HUVECs and CD34 positive cells, respectively.

Patients and blood collection

We studied 20 patients with KD (aged 8 months to 6 years; median 26 months; male/female = 15/5) and 10 healthy children (HC, aged 6 months to 4 years; median 23 months; male/female = 6/4). HC were recruited as follows: when parents required an examination to determine blood type (ABO) or a parent/child DNA-matching test for their children in our out-patient clinic, we asked the parents to provide an additional blood sample (1–2 ml) from their children. Informed consent was obtained from the parents of all patients and all HC. The present study was approved by the institutional review board. All KD patients were hospitalized at the National Defense Medical College Hospital between May 1998 and July 2000. The KD patients were enrolled within 7 days of the onset of illness, with day 1 defined as the first day of fever. All KD patients met the diagnostic criteria for KD established by the Japanese Kawasaki Disease Research Committee and were typical cases which fulfilled requirement of the criteria. All patients were scheduled to receive both aspirin (30 mg/kg/day) and intravenous immunoglobulin (i.v. IG, 2 g/kg). The initial course of i.v. IG therapy was started at days 5–7, and the second course of i.v. IG therapy was administered to a patient who eventually had aneurysms as a sequela.

Echocardiographic examinations

We evaluated the presence of coronary artery lesions (CAL) using two-dimensional echocardiographic examinations which were performed at 2–3-day intervals in the acute and subacute phases during hospitalization and at 2–3-month intervals in the convalescent phase at our out-patient clinic. The maximum internal diameters of both the right and left coronary arteries were determined. The arteries, which were greater than 2·5 mm in patients aged <24 months and greater than 3·0 mm in patients aged ≥24 months, were considered to demonstrate dilation. When the dilated arteries regressed to the normal range within 6 months after the onset of KD, these were considered to demonstrate transient dilation. The arteries, which were greater than 5·0 mm in all-aged patients, were considered to demonstrate aneurysms. Six of the 20 KD patients had CAL: five patients had transient dilation (diameter 3·0–4·0 mm) and one patient had bilateral aneurysms in left and right coronaries (maximum diameter 6·0 mm). Serial blood samples were obtained from all KD patients in the acute phase (before i.v. IG therapy on days 3–7), in the afebrile subacute phase (after i.v. IG therapy on days 9–16) and in the convalescent phase, when C-reactive protein (CRP) was negative, on days 22–37.

Immunomagnetic isolation and counting of CECs

The isolation of CECs was performed as described previously by Solovey *et al*. [6]. Briefly, after obtaining 1 ml of whole blood, the cells were immediately fixed by adding 0·25% paraformaldehyde for 10 min and they were then washed three times with phosphate-buffered saline (PBS). The samples were diluted 1 : 4 with a dilution buffer (Hanks's balanced salt solution without calcium and with 1 mM EDTA and 0·5% bovine serum albumin (BSA)). To isolate CECs from the sample buffer, the sheep antimouse IgG-carried magnetic beads were coated with the anti-EC murine MoAb (clone P1H12) according to the manufacturer's instructions. The diluted samples were mixed with 4 million anti-EC MoAb-coated beads per 1 ml of initial whole blood and then were incubated for 1 h at 4°C with gentle agitation. Next, the magnetic bead-bound cells were collected by using a magnetic concentrator (Dynal), resuspended with PBS containing 5% BSA, and cytocentrifuged equally onto four glass slides at 200 r.p.m. with a low acceleration by cytospin (Shandon, UK). The slide samples were stored at −20°C for 1–3 months by wrapping in aluminium foil, until the next-step experiments were carried out.

The detection and quantitative analysis of CECs were performed as described previously by George *et al*. [4]. Two smear slides were treated with May–Grünwald–Giemsa staining. The CECs were defined as cells demonstrating a cell size of larger than 30 μm in diameter and which were outlined by more than 10 attached beads (Fig. 1a,b). We determined the number of CECs (cells/ml) to be twice the total CEC number on two smear slides. Two observers counted the number of CECs blindly. After one observer counted the numbers of CECs in 10 slides, another reviewed them at random to test the interobserver agreement. We counted the numbers again on a different day and made sure that the numbers were repeatable.

Immunofluorescence of EC specific antigens

Smear slides were fixed in acetone, and were subsequently rehydrated in PBS. After treatment with PBS containing 2% BSA for 30 min, they were incubated with either murine MoAb against VE-cadherin or against E-selectin for 1 h and were then washed with PBS. They were next incubated with FITC-labelled-goat antibody against murine Ig for 1 h. After washing, they were counterstained with DAPI and mounted in glycerol. Finally, we observed the cells under fluorescence microscopy (Fig. 1c,d).

Detection and counting of EPCs

Two smear slides were incubated with PE-labelled MoAb against AC133 for 1 h, washed with PBS, and were then counterstained subsequently with DAPI and mounted in glycerol. The fluorescence smear slides were observed under confocal laser microscopy (LSM410, Carl Zeiss Inc., Oberkokken, Germany) (Fig. 1e,f). The number of EPCs (cells/ml) was counted in the same manner as the CECs as described above.

Statistical analysis

All data are expressed as the mean ± s.e. Any differences among the acute, subacute and convalescent phases in the same group were assessed by the Wilcoxon signed-rank test. Intergroup differences were analysed with the Mann–Whitney test. A P-value less than 0·05 was considered to be significant.

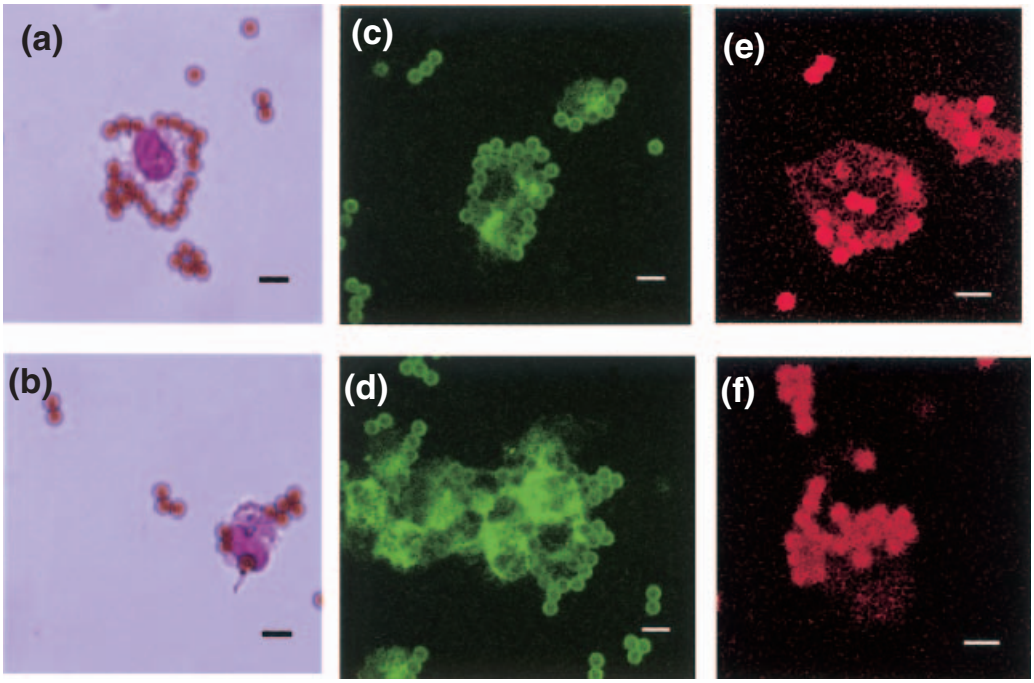

Fig. 1. Detection of CECs and EPCs isolated from the peripheral blood in KD patients. May–Grünwald–Giemsa staining revealed the cells which fulfilled the criteria (as described in Methods) of CECs (a) and did not (b), using P1H12 MoAb-coating magnetic beads. The CECs were confirmed by FITC-labelled MoAb against VE-cadherin (c) and E-selectin (d) under fluorescence microscopy. The cells were furthermore stained with PE-labelled AC133 MoAb and observed under confocal laser microscopy: a positive cell indicates EPC (e), while a negative cell indicates mature CEC (f). Scale bars indicates length of 10 μm.

RESULTS

The number of CECs (cells/ml) in HC was less than 6 cells/ml (3·2 ± 0·4/ml). The KD group was divided into two groups, including patients without CAL and with CAL (Fig. 2). Although the number of CECs had a wide scatter, the mean number of CECs was significantly higher ($P < 0·05$) in all (acute, subacute and convalescent) phases of KD than in HC (Fig. 2a). The mean number of CECs increased significantly from the acute (16·4 ± 2·1/ml) through the subacute (21·0 ± 2·1/ml) phases of KD and decreased significantly in the convalescent phase (9·1 ± 1·7/ml). Furthermore, the mean number of CECs was higher ($P < 0·05$) in KD patients with CAL than in those without CAL during all phases: acute phase, 23·0 ± 4·2 *versus* 13·6 ± 2·0/ml; subacute phase, 31·0 ± 3·1 *versus* 16·7 ± 1·7/ml; convalescent phase, 15·7 ± 4·9 *versus* 7·3 ± 0·9/ml, respectively.

Although no EPCs were observed in HC, EPCs were found in 11 of 20 KD patients, in six of 14 KD patients without CAL and in five of six KD patients with CAL (Fig. 2b). The proportion of EPCs in total CECs was 4·4± 1·2% (range 0–18%), and the mean number of EPCs was significantly higher in the subacute phase (1·4 ± 0·5/ml) than in either the acute (0·4 ± 0·2/ml) or the convalescent (0·8 ± 0·6/ml) phases. Therefore, the time course of EPCs appears to be different from that of CECs: the appearance of EPCs was a relatively late event, which follows the appearance of CECs. Furthermore, the mean number of EPCs during the subacute phase was significantly higher ($P < 0·05$) in KD patients with CAL (3·7 ± 1·1/ml) than those without CAL (1·0 ± 0·4/ml). In particular, in a KD patient with an aneurysm the number of CECs and EPCs tended to remain high even in the convalescent phase. The number of CECs and EPCs did not relate to either the clinical presentations or the laboratory data (CRP levels and WBC counts).

DISCUSSION

KD has been established pathologically as a type of systemic arteritis [20]. Although this disease causes EC injury, no specific serological markers of EC damage have been identified so far. The serum levels of von Willbrand factor, soluble selectins and soluble intercellular adhesion molecule-1 have been reported to increase in the acute phase of KD [21–23], but the expressions of these factors are not always restricted to ECs.

In the present study, the number of CECs increased during the clinical course of KD compared with HC. As all KD patients were treated with i.v. IG in the present study, it is unknown as to whether or not this therapy may have any effect on the CEC levels. KD patients with CAL had a peak number of CECs in the subacute phase, when CAL usually begins to be observed on echocardiographic examinations. Furthermore, as the proportion of EPCs in the total CECs were small in KD, the large population of these CECs was thought to be mature ECs, which may detatch from the vascular walls and therefore enter into the circulation. On the other hand, when we measured the number of CECs in five patients with anaphylactoid purpura, no increase in number was seen (data not shown). Therefore, the increased number of CECs may reflect the EC damage of KD, suggesting that they might be a useful marker of EC injury in KD. Although the mechanism of EC shedding could not be elucidated in the present

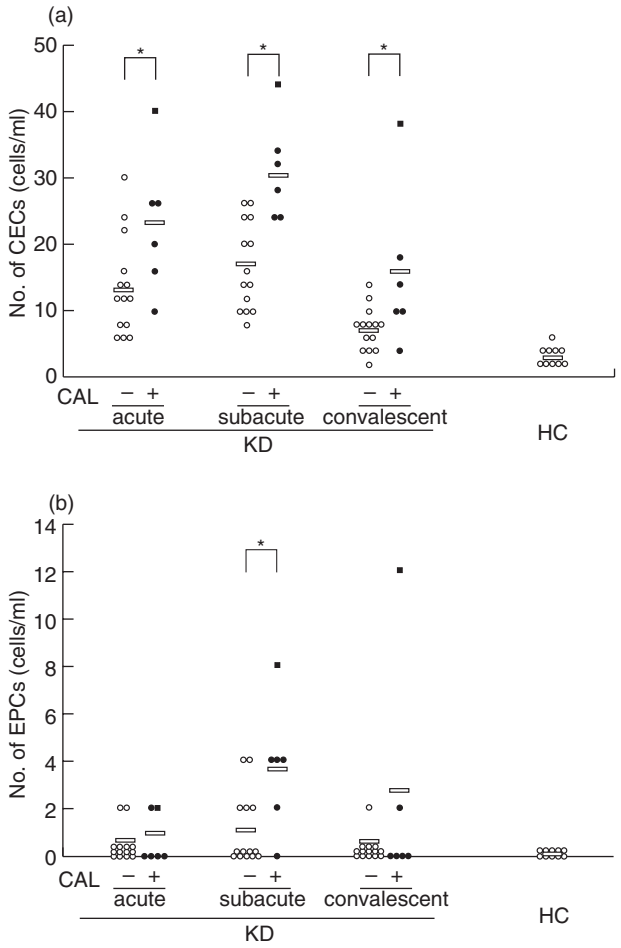

Fig. 2. The number of CECs (a) and EPCs (b) in KD patients and HC. Open bars indicate the mean value in each group. ○, KD patients without CAL; ●, KD patients with transient dilation; ■, a patient with aneurysm.*$P < 0{\cdot}05$.

study, it may be explained by several factors including cytokine and/or protease-induced detachment, an imbalance of pro/anti-angiogenic systems and an activation of apoptotic programmes [2].

EPCs are considered to be derived from a common haematopoietic precursor cell. EPCs have a highly proliferative potential, and have thus been suggested to contribute to vasculogenesis [12,13]. In addition, the plasma levels of vascular endothelial growth factor (VEGF), which accelerate neovascularization by mobilizing the EPCs into the circulation [24], have also been reported to increase in the acute and subacute phases of KD [25,26]. Recently, Suzuki *et al*. demonstrated immunohistochemically that an active remodelling process occurs in the CAL of KD [27]. In the present study, KD patients with CAL had a higher number of EPCs than those without CAL, and circulating EPCs increased with delayed kinetics following the increase in CECs. These findings indicate that the such EPCs are mobilized from the bone marrow into the circulation, thus suggesting that they might be involved in both the repair of EC damage and the potential microneovascularization observed in KD vasculitis. To investigate the significance of EPCs for CAL in KD, further immunohistochemical studies will be needed in future.

In summary, the number of CECs increased from the acute through the subacute phases of KD and was higher in patients with CAL than in those without CAL. Although the main origin of CECs may be mature ECs which have detached from the vessel walls, CECs also contain a small population of EPCs which originate from the bone marrow, especially in KD patients with CAL.

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